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Original Research Article

Dual Regulating Effect of Shaoyao-Gangcao-Tang on COX-2 Expression in Acute and Resolution Phases of Carrageenin-Induced Pleurisy in Rats

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Abstract

Purpose: *To investigate the effects and potential mechanisms of Shaoyao-Gangcao-Tang (SGT) on acute and resolution phases of carrageenin-induced pleurisy in rats.*

Methods: *To determine the effects of SGT at 2 h, Sprague-Dawley rats received injection of 0.2 ml of 1 % λ-carrageenin into the pleural cavity after treatment with 4.0, 13.3 and 40.0 g/kg SGT for three days. At 2 h after pleurisy induction, exudate volume, total cell number, prostaglandin E_2 (PGE_2) production and cyclooxygenase-2 (COX-2) protein expression were measured. To determine the effects at 48 h, the rats were treated with SGT at 24, 36 and 46 h after injection of λ-carrageenin into the pleural cavity, and the exudate volume, total cell number, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) production and COX-2 protein expression were evaluated.*

Results: *At 2 h after pleurisy induction, 13.3 and 40.0 g/kg SGT significantly decreased exudate volume by 34 ($p < 0.05$) and b 4 0% ($p < 0.01$), total cell number by 27 ($p < 0.05$) and 41 % ($p < 0.01$), PGE_2 production by 17 ($p < 0.05$) and 35 % ($p < 0.01$), as well as COX-2 protein expression by 21 ($p < 0.01$) and 43 % ($p < 0.01$) compared with control group treated with saline. At 48 h after pleurisy induction, 13.3 and 40 g/kg SGT also significantly decreased exudate volume by 36 ($p < 0.05$) and 55 % ($p < 0.01$), as well as total cell number by 31 ($p < 0.05$) and 43 % ($p < 0.01$), but markedly increased 15d-PGJ_2 production by 26 ($p < 0.05$) and 51 % ($p < 0.01$), as well as COX-2 protein expression by 50 ($p < 0.01$) and 100 % ($p < 0.01$) compared with control group.*

Conclusion: *The findings suggest that SGT has dual regulating effect in acute and resolution phases of inflammation, involving inhibiting acute inflammation through down-regulation of pro-inflammatory mediators, and promoting inflammatory resolution through up-regulation of pro-resolution mediators.*

Keywords: *Shaoyao-Gangcao-Tang, Cyclooxygenase-2, Prostaglandin E_2, 15-Deoxy-$\Delta^{12,14}$-PGJ_2, Inflammation*

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INTRODUCTION

Inflammation, which occurs in response to the harmful stimuli, is beneficial for our body to clean the harmful stimuli and restore the tissue structure and function. Therefore, the hallmark of a successful inflammatory response is not only the clearance of injurious stimuli, but also the restoration of normal physiology. If acute inflammation cannot resolve normally, it will switch to chronic inflammation resulting in ongoing tissue damage [1]. Thus, promoting

inflammatory resolution may be a promising method for the treatment of inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel diseases and asthma [2].

New evidence shows that the resolution of acute inflammation is a highly coordinated and active process which is regulated by endogenous pro-resolving mediators, such as lipoxin, protectin and 15d-PGJ_2 [3]. 15d-PGJ_2 was initially identified as a high-affinity natural ligand for peroxisome proliferator activated receptor gamma (PPARγ) [4]. It is now considered to exert its biological functions through PPARγ-dependent as well as -independent mechanisms. Studies have revealed that 15d-PGJ_2 can inhibit NF-κB [5] and AP-1 [6] activation, resulting in the reduction of pro-inflammatory mediators generation including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and COX-2, as well as the induction of apoptosis in activated macrophages [7] and myofibroblasts [8]. 15d-PGJ_2 also preferentially inhibits monocytes rather than polymorphonuclear leukocytes trafficking through the differential regulation of cell adhesion molecules and chemokines expression [9]. These data indicate that 15d-PGJ_2 can tightly regulate the resolution of acute inflammation.

As the key enzyme of regulating PGE_2 generation, COX-2 has been thought to be a pro-inflammatory mediator. However, Gilroy et al [10] suggested that COX-2 might promote resolution of inflammation for its contribution to 15d-PGJ_2 production. PGD_2 is a major product from the COX-catalyzed reaction. PGD_2 undergoes dehydration to yield biologically active PGs of the J_2 series, including PGJ_2, $\Delta^{12,\ 14}$-PGJ_2 and 15d-PGJ_2 [11]. Therefore, COX-2 has a dual role in inflammation progress, which is pro-inflammatory in acute phase and pro-resolving in resolution phase [12].

SGT, a widely used traditional Chinese formula, has been applied to various clinical symptoms associated with analgesia, anti-spasm in China for hundreds of years. Feng et al demonstrated that SGT could inhibit PGE_2 production [13], but the underlying mechanisms were not fully understood. Our previous work found that SGT markedly inhibited the pro-inflammatory mediators production and bone erosion in rats with adjuvant-induced arthritis (AIA) [14]. Considering that ongoing production of pro-inflammatory mediators and bone erosion in AIA resulted from abnormal resolution of inflammation, we thought that SGT might regulate the resolution of inflammation. To prove our presumption, in this study, we investigated the impact of SGT on exudate volume, total cell number, PGE_2 and 15d-PGJ_2 production, as well as COX-2 expression in acute and resolution phases of carrageenin-induced pleurisy in rats.

EXPERIMENTAL

SGT preparation

Shaoyao (*Paeonia lactiflora* Pall.) and Gancao (*Glycyrrhiza uralensis* Fisch.) were purchased from Chongqing Tongjunge Pharmacy (Chongqing, China) and were identified by Dr Ji-Fen Zhang, College of Pharmaceutical Sciences, Southwest University (Chongqing, China). SGT was prepared as we previously described [14]. The content of paeoniflorin in SGT extract used in this study was quantitatively analyzed by high performance liquid chromatography (HPLC) method as described previously [15]. In this study, SGT was found to contain 9.17 mg peoniflorin per g SGT extract.

Animals

Male Sprague-Dawley (SD) rats weighing 200 ± 20 g were purchased from Chongqing Medical University (Chongqing, China). All rats were housed in a temperature-controlled room (23 ± 2 °C) under a light/dark cycle with lights on from 7:00 am to 7:00 pm. They were allowed food and water *ad libitum*. The animals adapted to experimental environment for 1 week before experiments were carried out. All animal procedures were approved by the Ethical Committee in Animal Research of Chongqing Technology and Business University (approval no. 2011-6-6/CTBU). The procedures involving animals and their care conformed to international guidelines for the use of laboratory animals [16].

Carrageenin-induced pleurisy

0.2 ml of 1 %w/v λ-carrageenin (Sigma, MO, USA) suspended in saline was injected into the right pleural cavity of 30 rats under light ether anesthesia. Pleural exudate were collected at 2, 6, 18, 24 and 48 hours after injection. At each time point, pleural cavities of 6 rats washed out with 1.0 ml saline containing heparin (5 U/ml). Exudate and washing solutions were removed. The volume of the exudate was calculated by subtracting the volume of the washing solution (1.0 ml) from the total volume recovered. The cells were counted using a hemocytometer. The fluid was centrifuged at 1500 rpm for 5 min at 4 °C, the supernatant collected and stored at -70 °C for analysis of PGE_2 or 15d-PGJ_2. The pellets were subjected to total protein extraction for Western blot assay.

Animal treatment

To determine the effects at 2 h, rats were randomly divided into 4 groups with 10 rats in each group: (1) Control group, (2) 4 g/kg SGT group, (3) 13.3 g/kg SGT group, (4) 40 g/kg SGT group. Rats of groups (2), (3) and (4) groups were intragastrically treated with SGT twice a day for 3 consecutive days. Rats of group (1) group were treated with an equal volume of saline. One hour after the last treatment, pleurisy was induced by carrageenin injection into the right pleural cavity of rats of all groups. Two hours after this injection, inflammatory exudate was collected for analysis.

To determine the effects of SGT at 48 h, the rats were randomly divided into 4 groups as described above, and treated with SGT or saline at 24, 36 and 46 h after the injection of carrageenin. Inflammatory exudate was collected for analysis 48 h after this injection.

PGE_2 and 15d-PGJ_2 levels analysis

Inflammatory exudate was centrifuged at 1500 rpm for 5 min and total PGE_2 and 15d-PGJ_2 levels determined in the cell-free inflammatory exudate. PGE_2 was measured by a commercial radioimmunoassay kit (The General Hospital of the People's Liberation Army, Beijing, China). 15d-PGJ_2 was measured by a commercial enzyme-linked immunosorbent (ELISA) kit (Cayman Chemical, Michigan, USA). Assays were performed according to the manufacturer's instructions.

Western blot analysis

Inflammatory cell pellets were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS) [17] and EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). Equal amounts of protein samples were separated by SDS-PAGE electrophoresis and transferred onto PVDF membranes (Milipore, MA, USA). After blocked with 5 % nonfat milk in TBST buffer (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1 % Tween 20) for 1 hours at room temperature, PVDF membranes were incubated with anti-COX-2 antibody or anti-β-actin antibody (Santa Cruz, CA, USA) at 4 °C overnight. Then the membranes were incubated for 1 hours at room temperature with appropriate HRP-conjugated secondary antibody. The protein bands were detected with enhanced chemiluminescence reagents (Milipore, MA, USA). Chemiluminescent signals were adetected and analyzed by the *ChemiDoc XRS* imaging system (Bio-Rad, CA, USA).

Statistical analysis

All data were presented as mean ± standard error of mean (SEM). Significant differences between data were evaluated by ANOVA test using SPSS 16 software. $P < 0.05$ was considered as statistical significant.

RESULTS

In the carrageenin-induced pleurisy, exudate volume and total cell number increased quickly from 2 to 24 h after carrageenin injection, and thereafter declined from 24 to 48 h (Fig 1A and B). Maximal PGE_2 production occurred at 2 h, and declined from 2 to 48 h (Fig 1C). High COX-2 protein expression occurred at 2, 6 and 48 h during the carrageenin-induced pleurisy (Fig 1D). However, this protein could not be detected at 18 or 24 h. Interestingly, the expression of COX-2 at 48 h was much higher than that at 2 and 6 h. COX-2 protein expression at 48 h was approximately 41 and 63 % higher than at 2 and 6 h, respectively (Fig 1E).

At 2 h after injection of carrageenin, the levels of exudate volume and total cell number were 0.47 ± 0.06 ml and (7.27 ± 0.67) × 10^6 cells, respectively. Doses of 13.3 and 40 g/kg SGT significantly reduced the exudate volume to 0.32 ± 0.06 ($p < 0.05$) and 0.28 ± 0.03 ml ($p < 0.01$), equivalent to reduction of about 34 and 40% compared with Control group, respectively (Fig 2A). For total cell number, 13.3 and 40 g/kg SGT significantly reduced the level to (5.33 ± 0.32) × 10^6 ($p < 0.05$), (4.3 ± 0.4) × 10^6 cells ($p < 0.01$), i.e., a reduction of about 27 and 41 %, respectively, compared with Control group (Fig 2 B).

At 2 h after injection of carrageenin, the level of PGE_2 in pleural exudate was 2111 ± 182 pg/ml. 13.3 and 40 g/kg but SGT significantly reduced the level to 1745 ± 63 ($p < 0.05$) and 1370 ± 193 pg/ml ($p < 0.01$), respectively (Fig 3A), a reduction of about 17 and 35 %, respectively, compared with Control group, respectively. Dose of 13.3 and 40 g/kg SGT also significantly reduced COX-2 protein expression by approximately 21 ($p < 0.01$) and 43 % ($p < 0.01$), respectively, compared with control group (Fig 3B and C).

The levels of exudate volumes and total cell number were 0.22 ± 0.05 ml and (30 ± 4.36) × 10^6 cells, respectively, at 48 h after injection of carrageenin. Doses of 13.3 and 40 g/kg SGT significantly reduced exudate volume to 0.14 ±

0.02 ml ($p < 0.05$) and 0.1 ± 0.01 ml ($p < 0.01$), respectively, equivalent to a reduction of approximately 36 and 55 %, respectively, compared with Control group (Fig 4A). Doses of 13.3 and 40 g/kg SGT also significantly reduced total cell number to (20.67 ± 2.08) × 10^6 ($p < 0.05$) and (17 ± 2) × 10^6 cells ($p < 0.01$), which amounts to a reduction of about 31 and 43 %, respectively, compared with Control group (Fig 4 B).

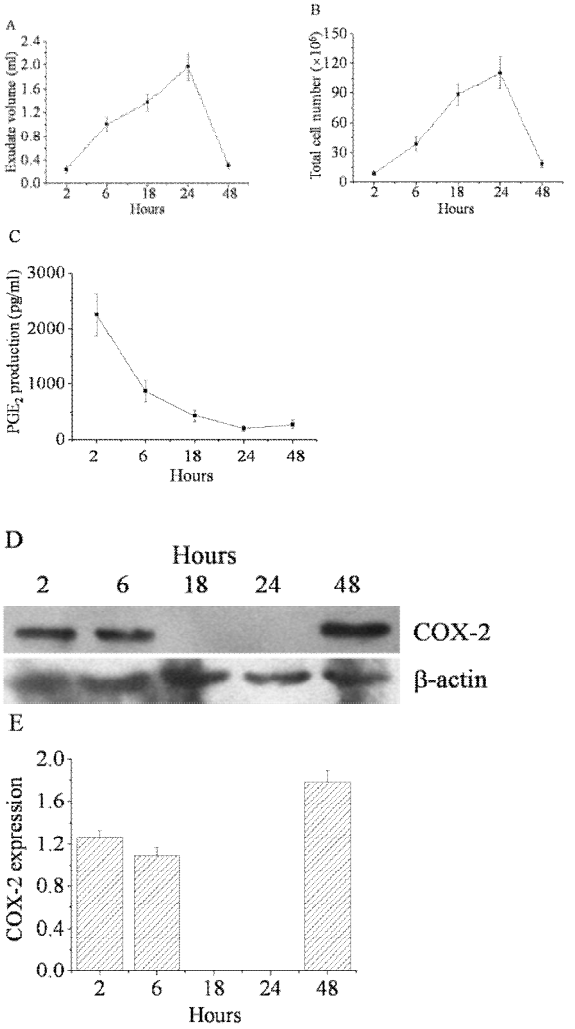

Fig 1: Inflammatory trend in carrageenin-induced pleurisy. Exudate volume (A), total cell number (B) and PGE_2 production (C) at 2, 6, 18, 24, 48 h after injection of carrageenin (n = 6). D: Western blot analysis of COX-2 protein expression at 2, 6, 18, 24, 48 h after injection of carrageenin. β-actin was used as loading control. The result presented was representative of four independent experiments. E: Bar charts show quantitative evaluation of COX-2 bands by densitometry from four independent experiments.

Forty eight hours after injection of carrageenin, the level of 15d-PGJ_2 in pleural exudate was 752 ± 55 pg/ml. Doses of 13.3 and 40 g/kg SGT significantly increased the level of 15d-PGJ_2 to 948 ± 67 ($p < 0.05$) and 1139 ± 82 pg/ml ($p < 0.01$), respectively, which amount to increase of about 26 and 51 %, respectively, compared with control group (Fig 5A). Doses of 13.3 and 40 g/kg SGT also significantly increased COX-2 protein expression by > 50 ($p < 0.01$) and 100 % ($p < 0.01$), respectively, compared with control group (Fig 5 B, C).

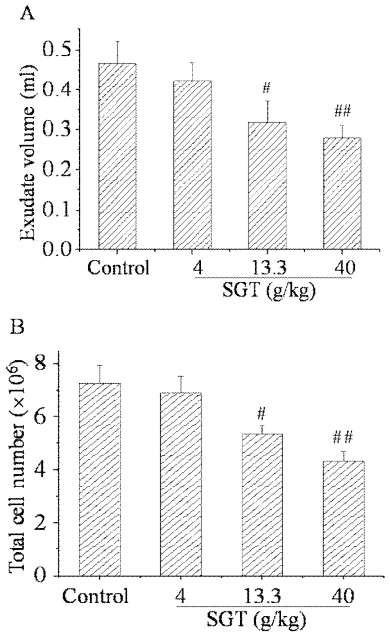

Fig 2: Effect of SGT on exudate volume (A) and total cell number (B) at 2 h after injection of carrageenin (n = 10). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with control group

DISCUSSION

In the present study we found that SGT significantly decreased the exudate volume, total cell number, prostaglandin E_2 (PGE_2) production and COX-2 protein expression at 2 h of λ-carrageenin-induced pleurisy. We also found that SGT markedly decreased the exudate volume and total cell number 48 h after pleurisy innduction. Interestingly, we found that 15d-PGJ_2 production and COX-2 protein expression increased 48 h after SGT administration induced pleurisy. These data suggest that SGT has dual regulating action on acute and resolution phases of inflammation, involving inhibiting the acute inflammation and promoting the inflammatory resolution.

Carrageenin-induced pleurisy is commonly used in studies of acute inflammation and inflammatory resolution [10]. In this study, we found that exudate volume and total inflammatory cell number peaked at 24 h and resolved by 48 h in carrageenin-induced pleurisy in rats. Another study demonstrated that cellular infiltrate was initially dominated by neutrophil granulocytes up to 12 h [10]. With the increasing amount of mononuclear cells as inflammation progresses, neutrophil granulocytes were

replaced by migrating monocytes, which differentiated into macrophages. These monocytes became dominant up to resolution at 48 h. Since there was a fast alternation of acute inflammation and inflammatory resolution during 48 h in carrageenin-induced pleurisy, we used this model for our investigations.

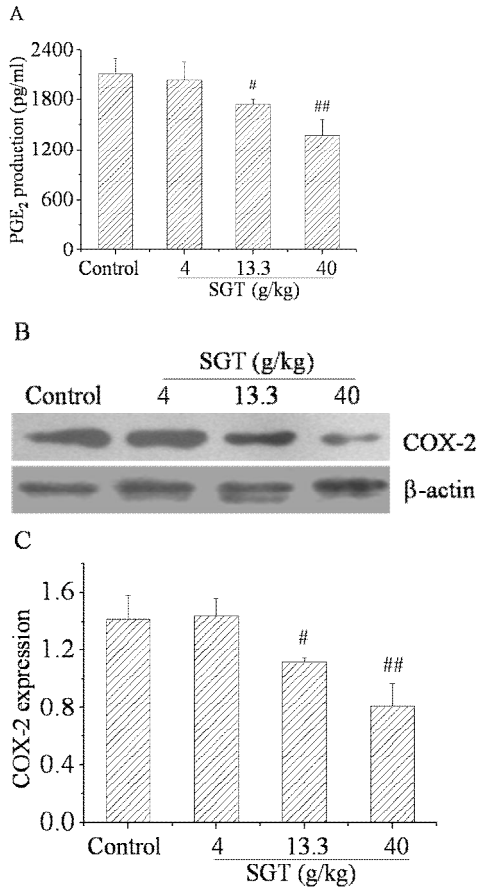

Fig 3: Effect of SGT on PGE_2 production and COX-2 protein expression at 2 h after injection of carrageenin. A: Effect of SGT on PGE_2 production at 2 hours after injection of carrageenin (n = 10). B: Effect of SGT on COX-2 protein expression by Western blot analysis at 2 hours after injection of carrageenin. β-actin was used as loading control. The result presented was representative of four independent experiments. C: Bar charts show quantitative evaluation of COX-2 bands by densitometry from four independent experiments; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with control group.

First, we investigated the impact of SGT on acute phase of carrageenin-induced pleurisy. We selected the two indices of exudate volume and total cell number because they were positively associated with the severity of inflammation [18]. We selected the 2 h of pleurisy because, at this time, we discovered that there existed considerable COX-2 protein expression, and maximal PGE_2 production. We found that, at 2 hours of carrageenin-induced pleurisy, doses of 13.3 and 40 g/kg SGT significantly reduced t exudate volume and total cell number in exudate in a dose-dependent manner. However, 4 g/kg did not show statistically inhibitory effects. These results suggest that SGT could inhibit the acute inflammation. Considerable amounts of PGE_2, mainly synthesized by COX-2, is generated at sites of inflammation where it acts as a potent vasodilator and synergistically with other mediators such as histamine and bradykinin, resulting in enhancement in vascular permeability and edema [19]. The results indicate that SGT might inhibit the acute inflammation through suppressing the COX-2 protein expression and PGE_2 production.

Since SGT administration did not interfere with the progress of acute inflammation, we could directly and exactly discover the effects and mechanisms of SGT on inflammatory resolution. SGT significantly decreased the exudate volume and total cell number at 48 hours of carrageenin-induced pleurisy, indicating that SGT promotes inflammatory resolution. Interestingly, accompanying the reduction of PGE_2, COX-2 protein expression was clearly enhanced at 48 h. Although this seems implausible, the following mechanism may make it more likely: In the resolution phase of inflammation, COX-2 catalyzes the production of cyclopentenone prostaglandins of the J-series (15d-PGJ_2) in macrophages, which act as a pro-resolving mediator in inflammation [20].

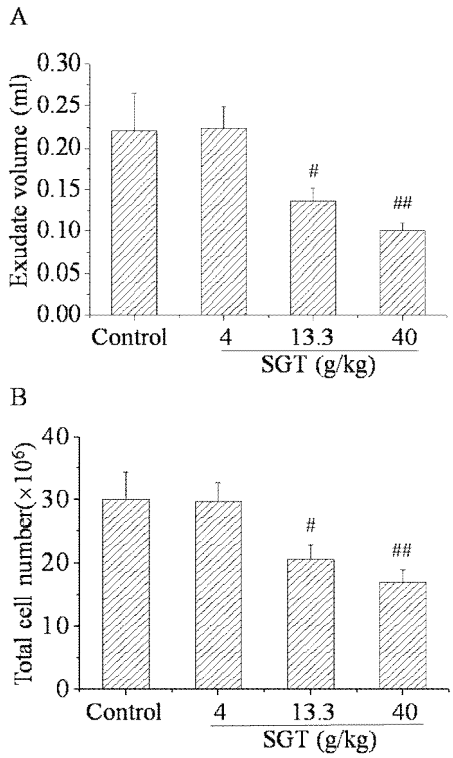

Fig 4: Effects of SGT on exudate volume (A) and total cell number (B) at 48 h after injection of carrageenin (n = 10). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with control group

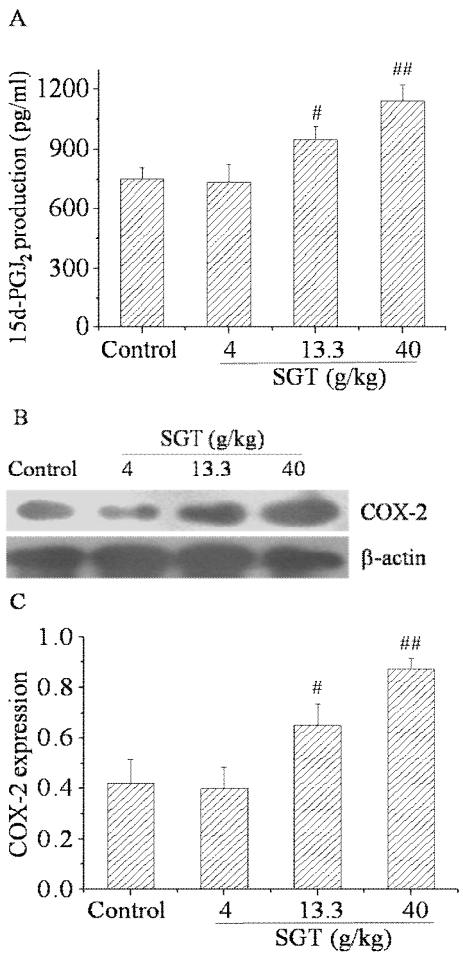

Fig 5: Effects of SGT on 15d-PGJ_2 production and COX-2 protein expression at 48 h after injection of carrageenin. A: Effect of SGT on 15d-PGJ_2 production at 48 hours after injection of carrageenin (n = 10). B: Effect of SGT on COX-2 protein expression by Western blot analysis at 48 hours after injection of carrageenin. β-actin is used as loading control. The result presented was representative of four independent experiments. C: Bar charts show quantitative evaluation of COX-2 bands by densitometry from four independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with control group

CONCLUSION

SGT has dual regulating action on acute and resolution phases of carrageenin-induced pleurisy, involving inhibiting acute inflammation and promoting inflammatory resolution. The mechanisms of this action may decrease COX-2 protein expression and PGE_2 production in acute phase of inflammation, as well as increase COX-2 protein expression and 15d-PGJ_2 production in the resolution phase of inflammation. To our knowledge, this study is the first one to explore the dual regulating action of traditional Chinese formula on acute inflammation and inflammatory resolution. These findings suggest that SGT is a promising anti-inflammatory drug candidate that requires further investigation.

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